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学位授与の題目	Signal transduction of an insect steroid hormone, 20-hydroxyecdysone, in 20E-induced programmed cell death of the silkworm anterior silk glands （カイコガ前部絹糸腺の 20E 誘導性予定細胞死における昆虫ステロイドホルモン、20 ヒドロキシエクジソン、のシグナル伝達）
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Abstract

The insect steroid hormone 20E induces programmed cell death of larva-specific tissues at pupal metamorphosis. In the silkworm *Bombyx mori*, the anterior silk gland undergoes cell death in response to the metamorphic peak titer of ecdysteroids *in vivo* and also to 20E *in vitro*. Although 20E elicits early gene activation, an additional 20E stimulus is required for completion of cell death. This additional stimulus leads caspase-3-like protease activation, indicating that 20E also elicits its effects through a nongenomic mechanism. Studies using various inhibitors and antagonists have shown that cell shrinkage is under the control of 20E genomic action, while nuclear condensation, nuclear fragmentation and DNA fragmentation are under the 20E nongenomic action that begins with 20E-binding to the putative membrane-bound ecdysone receptor (mEcR). The present pharmacological studies indicate that the mEcR is probably a G-protein-coupled receptor. Activation of GPCR is followed by a signaling pathway comprising of PLC/ IP_3R/ Ca^{2+}/PKC/caspase-3-like protease, which leading DNA and nuclear fragmentation. Nuclear condensation is out of this signaling pathway. It is regulated by signaling of calmodulin/calmodulin-dependent protein kinase II (CaMKII), but CaMKII activation is independent of intracellular Ca^{2+} elevation.

Introduction

The effects of steroid hormones to modulate many physiological processes including programmed cell death (PCD). During this period, unnecessary larval specific tissues will be degenerated before the pupation occurs. 20E is an insect steroid hormone which induces PCD in anterior silk gland (ASGs) of the silkworm, *Bombyx mori*. The mode of action of 20E are known to acts by binding to its heterodimeric hormone receptor, ecdysone receptor (EcR), and partner molecule, ultraspiracle (USP). In addition to genomic action of 20E, nongenomic action of 20E has been clearly shown by using cycloheximide (CHX).

In the nongenomic pathway, Ca^{2+} acts as a second messenger to activate protein kinase C (PKC) and caspase-3-like protease, which leads to DNA and nuclear fragmentation (Iga *et al.*, 2007), but the signaling pathway leading to the increase in intracellular Ca^{2+} concentration remained to be dissolved. Various inhibitors were used to search for the components involved in the 20E signal transduction pathways, and I will describe about the signaling pathway from the putative mEcR to elevation of cytosolic Ca^{2+} concentration. However, the steps leading to a concomitant buildup of intracellular Ca^{2+} are unknown. I employed pharmacological tools to identify the components of this pathway.

Material and Methods

Tissue culture

ASGs were dissected under dry conditions, then rinsed with Grace's insect cell culture medium (Gibco BRL., Rockville, MD) or Ca^{2+}-free Grace's medium (Grace, 1962), and cultured individually in 0.3 ml of medium using 24-well plates (Greiner Bio-One, Frickenhausen, Germany) at 25 °C for 120 h. Cellular morphology was recorded daily and used to note the progression of PCD according to the PCD scoring system described by Terashima *et al.* (2000).

Staining of nuclei

In order to examine the occurrence of nuclear condensation and nuclear fragmentation, I visualized nuclei with DAPI staining. ASGs were fixed in 4% formaldehyde for 30 min followed by washing with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 7.4), and then incubated in PBS containing 0.1 μg/ml DAPI at 25°C in the dark for 10 min. The ASGs were then washed with PBS and observed under a fluorescence microscope using a UV excitation filter (BX-50; Olympus, Tokyo, Japan). All images were processed with Photoshop® (Adobe Systems Inc., San Jose, CA).

Detection of DNA fragmentation

ASGs were homogenized and mixed with DNA extraction buffer (10 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA–NaOH, and 0.1% SDS, pH 8.0) on ice, and then the homogenates were treated with RNase (20 μg/ml, 37 °C for 30 min) and proteinase K (100 μg/ml, 50 °C for 60 min). DNA was extracted by standard procedures using phenol–chloroform and chloroform, and 1 μg DNA of each sample was loaded onto a 2% agarose-S gel for electrophoresis. The gels were stained with ethidium bromide (EtBr) and observed under a UV transilluminator connected to a CCD gel scanning system (Bioinstrument; Atto Corp., Tokyo, Japan).

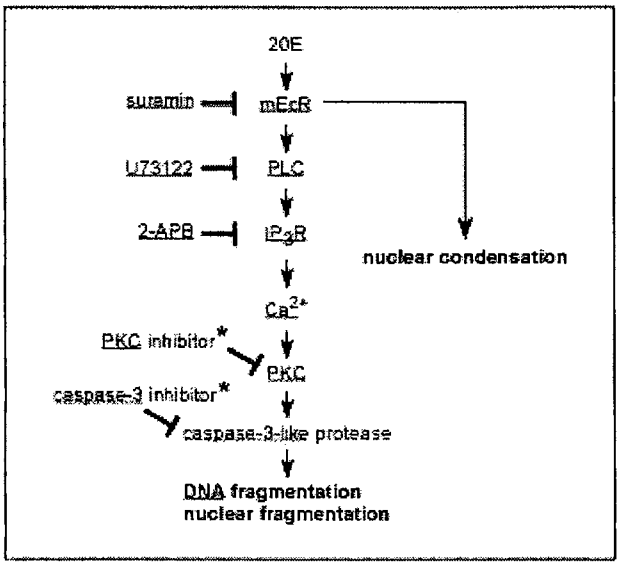
Results

The T-type Ca^{2+} channel blocker inhibited 20E-induced nuclear and DNA fragmentation; in contrast, PCD was induced by 20E in Ca^{2+} -free medium, indicating that the source of Ca^{2+} is an intracellular reservoir. The IP_3R antagonist inhibited nuclear and DNA fragmentation, suggesting that the endoplasmic reticulum may be the Ca^{2+} source. Finally, the GPCR and PLC inhibitors effectively blocked nuclear and DNA fragmentation. This indicate that 20E increases the intracellular level of Ca^{2+} by activating IP_3R, and that this effect may be brought about by the serial activation of GPCR, PLC, and IP_3. In addition, the inhibitors of CaM and Ca^{2+}/CaM-dependent kinase II effectively inhibited nuclear condensation in addition to nuclear and DNA fragmentations.

Discussion and Conclusions

In the 20E-induced PCD in *B. mori* ASGs, Ca^{2+} functions as the second messenger in the activation of PKC/caspase-3-like protease pathway, which causes nuclear and DNA fragmentation (Iga *et al.*, 2007). The summary table of inhibitors effects on 20E-induced PCD is presented in Table 2. The present study suggests a possible nongenomic pathway connecting the binding of 20E to putative mEcR with a rise in cytosolic Ca^{2+} as shown in the summary figure.

The present results indicate that ER is a possible intracellular Ca^{2+} storage site. 20E induced DNA and nuclear fragmentation irrespective of the presence or absence of Ca^{2+} in the culture medium, and an IP_3R antagonist, 2-APB, strongly inhibited the induction of DNA and nuclear fragmentation by 20E. Since IP_3R is localized on the ER membrane, a rise in the cytosolic Ca^{2+} level may be ER-dependent. Accordingly, 20E may induce Ca^{2+} mobilization from the ER, followed by PKC and caspase-3-like protease activation, which causes DNA and nuclear fragmentation. Although Ca^{2+} ionophore was incapable of inducing cell shrinkage, and PKC inhibitor did not inhibit the shrinkage (Iga *et al.*, 2007), 2-APB successfully suppressed cell shrinkage in addition to DNA and nuclear fragmentation. These results indicate that the signaling pathway up to cell shrinkagediffers from that leading to DNA fragmentation and nuclear fragmentation, but the solstice is not clear. At least, 2-APB may not block only Ca^{2+} mobilization through IP_3R but also inhibit an unknown factor(s) in the signaling pathway leading to the cell shrinkage.



* After Iga *et al.* (2007)

Schematic representation of possible 20E signaling in the 20E-induced PCD of Bombyx anterior silk glands using various inhibitors.

20E elicits its nongenomic effects through at least two signaling pathways, one leading to nuclear condensation and the other to DNA and nuclear fragmentation (Iga *et al.*, 2007). The present study provides pharmacological evidence of a signaling pathway from GPCR to PLC, which is subsequently separated into two parts. One is the pathway responsible for the regulation of DNA and nuclear fragmentation and may involve a signaling through IP_3/IP_3R, followed by a rise in the cytosolic Ca^{2+} level and PKC/caspase-3-like protease activation. The other should be for nuclear condensation is suggested to be in volved with CaMKII. CaMKII activation commonly mediates extracellular signal transduction in the cell death sequence, and Ca^{2+} /CaM-independent activation is brought about through autophosphorylation of CaMKII, although the initial phase of autophosphorylation requires mobilization of intracellular Ca^{2+}. In the 20E-induced cell death of *Bombyx* ASGs, a Ca^{2+} ionophore may not activate CaMKII, since it does not mimic 20E by inducing nuclear condensation (Iga *et al.*, 2007). Therefore, Ca^{2+} -independent, calmodulin-dependent CaMKII activation might be involved in induction of nuclear condensation by 20E, although the initial phase of the activation remained to be seen.

Reference

Iga, M., Iwami, M., Sakurai, S., 2007. Nongenomic action of an insect steroid hormone in steroid-induced programmed cell death. Molecular and Cellular Endocrinology 263, 18–28.

学位論文審査結果の要旨

昆虫の後胚発生は、昆虫ステロイドホルモンである20-ヒドロキシエクジソン（20E）により支配されている。本論文提出者は、20Eによる前部絹糸腺の予定細胞死を研究対象とし、20Eの核受容体を介さないnongenomic作用におけるシグナル伝達系を、薬理学的手法を用いて研究した。種々のアゴニストとアンタゴニストを使った実験結果は次の経路の存在を示唆した。すなわち、20EはG-タンパク質共役型受容体（GPCR）に結合し、次いでphospholipase-Cb（PLCb）を活性化する。活性化PLCbはinositol-3-phosphate（IP_3）を産生し、IP_3は小胞体膜上でCa^{2+}チャンネルとして機能するIP_3受容体を開け、細胞内Ca^{2+}濃度を上昇させ、protein kinase C/caspase 3-like proteaseの系が活性化し、核の断片化とDNA断片化が起きること。一方、この系を阻害しても細胞死に付随して生じる核凝縮は阻害されず、カルモジュリン及びカルモジュリン依存性キナーゼ（CaMK）により調節されていること。また、カルモジュリン/CaMKの活性化は直接にはCa^{2+}の細胞内濃度上昇に依存せず、この系を阻害すると核とDNAの断片化も阻害されること等を発見した。これらの結果は、20Eのnongenomic作用を仲介する経路が少なくとも2種類あり、これらが調和的に作動することにより、細胞死が完了することを示した。よって、本研究は20Eのnongenomic作用を理解する上で大きく寄与するものであり、本論文は学位（学術）を授与するに足るものである。